DIFFERENTIATION OF B. COLI AND B. AEROGENES ON A SIMPLIFIED EOSIN-METHYLENE BLUE AGAR

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For confirming the presumptive test for B. coli the mediums most frequently employed are litmus lactose agar and fuchsin sulphite (Endo) agar. It is becoming more apparent that the coli-like forms may be divided into two groups which are closely correlated with the source. One group (B. coli) is characteristic of fecal origin; the other (B. aerogenes and B. cloacae) is rare in feces, but constitutes the prevailing coli-like form in the soil and on grains. The standard litmus lactose and Endo agar may be employed to a slight extent for the differentiation of B. coli and B. aerogenes, but the differences between these types on these mediums (particularly L.L.A.) are not very clear-cut nor distinct. Better results are obtained with a modified Endo agar described elsewhere. A very excellent differentiation between the B. coli and B. aerogenes types has been obtained on a modification of eosin-methylene blue agar first described by Holt-Harris and Teague for the isolation of the typhoid group from feces. The medium is prepared in the following manner:

Distilled water	1000 c c
Peptone (Difco)	10 gm.
Dipotassium phosphate	2 gm.
Agar	15 gm.

Boil ingredients until dissolved and make up any loss due to evaporation.

Place measured quantities in flasks and sterilize at 15 lbs. for 15 minutes.

Just prior to use add to each 100 c c of the melted agar, prepared as above, the following constituents:

Sterile (20%) lactose solution	1 gm. or 5 c c
Aqueous (2%) eosin (yellowish) solution	2 c c
Aqueous (2%) methylene blue solution	2 c c

Pour medium into petri dishes, allow them to harden in incubator and inoculate in the ordinary way. Smearing the surface with a glass rod seems preferable to the streaking method sometimes employed.

There is no adjustment of reaction and filtration of medium is not necessary.

B. typhosus and members of intermediate group also grow well on this medium producing transparent, colorless, or slightly amber colonies that are about one-half the size of B. coli.

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	B. coli	B. aerogenes.
Size:	Well isolated colonies are 3-4 mm. in diameter.	Well isolated colonies are larger than coli; usually 4-6 mm. in diameter or more.
Confluence:	Neighboring colonies show little tendency to run together.	Neighboring colonies run together quickly.
Elevation:	Colonies slightly raised; surface flat or slightly concave, rarely convex.	Colonies considerably raised and markedly convex; occasionally the center drops precipitately.
Appearance by Transmitted Light:	Dark almost black centers which extend more than ¾ across the diameter of colony; internal structure of central dark portion difficult to discern.	Centers deep brown; not as dark as B. coli, and smaller in proportion to the rest of the colony. Striated internal structure often observed in young colonies.
Appearance by Reflected Light:	Colonies dark, button-like, often concentrically ringed with a greenish metallic sheen.	Much lighter than B. coli.. Metallic sheen not observed except occasionally in depressed center when such is present.

RESULTS WITH PURE CULTURES

A number of pure cultures were employed to test the value of this medium for the differentiation of B. coli, B. aerogenes, and members of the typhoid and paratyphoid groups.

Of 22 cultures of B. aerogenes all but 3 gave the characteristic reactions. Of these 3 cultures, 1 resembled B. coli on the eosin-methylene blue agar, another failed to produce a black center, and the 3rd showed a slight metallic lustre, but did not resemble B. coli closely.

Of 35 cultures of B. coli tested, 29 were typical. Six did not show a distinct metallic lustre, but were typical in other respects.

There were 23 strains of B. cholerasuis, B. paratyphosus, and B. typhosus tested. One strain of B. paratyphosus A did not grow. All other strains of the intermediate group developed typical transparent colonies.

RESULTS OBTAINED WITH WATER SAMPLES

The differentiation of pure strains seemed to be so marked and distinct that it was thought the medium might be employed for confirmation of the presumptive test for B. coli, and that it might be pos-

sible to differentiate B. coli from B. aerogenes simultaneously with confirming the presumptive test. For this purpose the following experiment was carried out.

Seven samples of water from different parts of the Iowa river, one of sewage, one of a small creek, and one from a stagnant body of water were plated out directly on litmus lactose agar and inoculated into lactose broth. After 24 hours' incubation 10 acid colonies were fished from the litmus lactose agar plates of each sample for further study. The lactose broth tubes were plated out after 48 hours' incubation onto eosin-methylene blue agar and onto litmus lactose agar. After 24 hours' incubation 10 colonies were fished from these litmus lactose agar plates of each sample for further observation. From the eosin-methylene blue plates made from the preliminary lactose broth tubes colonies which resembled B. coli or B. aerogenes were fished and tentatively designated as such, with a view to determining the accuracy and reliability of the plate differentiation.

All colonies fished from litmus lactose agar were reinoculated into lactose broth and after 24 hours' incubation were plated out on eosin-methylene blue agar. From each plate was picked a well isolated colony which was designated as B. coli or B. aerogenes. These designations were then checked by growing the organisms in Clark and Lubs medium and testing with the methyl-red and Voges-Proskauer reactions.

CULTURES OBTAINED DIRECTLY FROM LITMUS LACTOSE AGAR

Of the 10 water samples examined 1 did not show acid colonies by direct plating on litmus lactose agar. Of the 90 acid colonies fished, 3 were found to be lactose nonfermenters. Thirty-three cultures were regarded, from their appearance on eosin-methylene blue agar, as B. aerogenes. Of these, 24 (72%) gave the Voges-Proskauer reaction. Seven cultures were diagnosed tentatively as questionable but probably B. aerogenes but none of these gave a Voges-Proskauer reaction.

Eight cultures were regarded as questionable, but probably B. coli, of which 6 (75%) did not give the Voges-Proskauer reaction. Thirty-nine cultures were designated from their appearance on eosin-methylene blue agar, as B. coli, and all were confirmed, as none gave the Voges-Proskauer reaction.

Of 40 cultures which were regarded tentatively as B. aerogenes or probably B. aerogenes 24 (60%) were correct. Of 47 cultures regarded as B. coli or probably B. coli, 45 (95.8%) were correct.

CULTURES OBTAINED FROM LITMUS LACTOSE AGAR AFTER PRELIMINARY ENRICHMENT IN LACTOSE BROTH

After elimination of a few strains which proved to be other than coli forms, 85 cultures which were isolated from litmus lactose agar plates made from the lactose broth preliminary enrichment tubes were

smeared onto eosin-methylene blue agar for differentiation. One organism was regarded as probably B. aerogenes and on confirmation proved to be B. coli. Of 35 organisms recorded as B. aerogenes 34 (97%) gave the Voges-Proskauer reaction. Forty-nine organisms were tentatively designated as B. coli or probably B. coli and all proved to be negative for the Voges-Proskauer reaction.

With organisms isolated from this group then, 34 out of 36 cultures regarded as B. aerogenes were confirmed as such while every one of 49 strains regarded as B. coli was correct.

CULTURES OBTAINED FROM EOSIN-METHYLENE BLUE AGAR AFTER PRELIMINARY ENRICHMENT IN LACTOSE BROTH

Fifty-two cultures were fished, 26 of which were regarded as B. aerogenes and the remaining as B. coli. Of the 26 supposedly B. aerogenes strains all gave the Voges-Proskauer reaction. Two of the strains regarded as B. coli also gave the Voges-Proskauer reaction. Thus 100% of the B. aerogenes strains and 92.4% of the B. coli strains were correctly differentiated on eosin-methylene blue agar.

Results on all cultures isolated may be summarized as follows:

Tentatively regarded as B. coli from appearance of eosin-methylene blue agar	122
Correctly designated as indicated by negative Voges-Proskauer reaction	118
Per cent. confirmed	96.9
Tentatively regarded as B. aerogenes from appearance on eosin-methylene blue agar	102
Correctly designated as indicated by positive Voges-Proskauer reaction	84
Per cent. confirmed	82.4

CORRELATION OF VOGES-PROSKAUER AND METHYL-RED REACTION

In previous work a marked correlation was observed between the Voges-Proskauer and methyl-red reactions. Strains of coli-like forms which were acid to methyl-red characteristically did not give a test for acetyl-methyl-carbinal (V-P negative); while those reacting alkaline to methyl-red gave a positive Voges-Proskauer test. These observations were confirmed by Greenfield,[1] Hunter,[2] Clark,[3] Hutton,[4] Rettger and Burton[5] and others.

In this group of strains studied a similar correlation was observed. The relation between the Voges-Proskauer and methyl-red reactions is indicated in the following table:

	Methyl-red		
V. P.	+	N.*	—
+	2	2	84
—	121	13	2

* In previous work neutral reactions to methyl-red have been grouped with the acid strains.

It is seen from the table that there is an excellent correlation between the two reactions. Placing the neutral reacting strains with the acid group as was done in previous studies, we find that 84 (97.8%) of 86 methyl-red negative strains give the V. P. reaction; while of 136 strains not giving the V. P. reaction 134 (98.7%) were acid or neutral to methyl-red.

In this series of cultures the V. P. reactions were more clear-cut than the methyl-red test but we have worked with collections in which the reverse was true. It seems best to employ both the V. P. and methyl-red tests and to repeat if the results do not agree.

It is interesting to note that of 87 cultures isolated from litmus lactose agar plates made directly, 29.9% proved to be B. aerogenes; whereas of 85 cultures isolated from litmus lactose agar plates made after preliminary enrichment for 48 hours in lactose broth, 40% proved to be B. aerogenes. This indicates the correctness of the contention of Race and others that preliminary enrichment tends to an overgrowth of B. aerogenes types.

Organisms other than B. coli and B. aerogenes grow quite well on this medium and several have been observed to produce small blue centers; but the appearance is so distinct from B. coli and B. aerogenes that once having observed the true types there should be no mistake. Just what these other forms are has not been determined but several have been isolated and will be reported on in a future report. They produce very small colonies with pinpoint light blue or delf-blue centers. The color is very different from the brownish black appearance of the B. coli and B. aerogenes types. Perhaps the introduction of some inhibitory dye into the medium will make it even more reliable for the isolation and differentiation of B. coli and B. aerogenes and confirmation of the presumptive test.

[1] Jour. Infect. Dis., 1916, 19, p. 647.
[2] Jour. Bacteriol., 1917, 2, p. 585.
[3] Jour. Biol. Chem., 1917, 30, p. 209.
[4] Jour. Infect. Dis., 1916, 19, p. 606
[5] Ibid., 21, p. 162.